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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 7 - 1975
INSTITUTE

Application for Research Grant
(Use extra pages as needed)

Date 6/30/75

1. Principal Investigator (give title and degrees).

Mario D. Aceto, Ph.D., Associate Professor of Pharmacology

2. Institution & address:

Medical College of Virginia
Virginia Commonwealth University
Health Sciences Division
MCV, Box 726
Richmond, VA 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Antinicotinic Effects and Antianxiety Agents

5. Proposed starting date: Jan. 2, 1976

6. Estimated time to complete. 2 years

7. Brief description of specific research aims:

1) Determine the relative localization of nicotine-^{14}C over a wide dose range in selected rat brain areas.

2) Determine the subcellular distribution of nicotine-^{14}C in the rat brain.

3) Study the effects of antianxiety agents such as librium and meprobamate upon nicotine-^{14}C localization.

4) Attempt to ascertain a functional role for the central nicotinic nervous system as it relates to the mechanism of action of antianxiety agents.

1003540748

8. Brief statement of working hypothesis:

Although it has been shown that cholinergic receptors in the central nervous system (CNS) may be either nicotinic or muscarinic (Eccles, 1964), little work has been done on the localization of nicotine in the brain and on its subcellular distribution (Larson and Silvette, 1964, 1968, 1971). Even less is known about the role of the nicotinic nervous system and its interactions with CNS drugs. Studies by Hansson and Schmiterlow (1962) have shown that soon after the administration of nicotine-methyl-^{14}C in mice, very high concentrations of nicotine appeared in the CNS. Studies by the investigator (Aceto, 1967) showed that for the intraventricular (intracerebral) route of administration that a direct relationship between ganglion blocking potency and blocking of nicotine extensor convulsions existed and that the site of nicotine extensor convulsions is central in origin and is associated with brain areas near the ventricles. Later in 1971, Benesova and Nahunek reported a correlation between the degree of antinicotine convulsant activity and the clinical efficiency of antidepressants in agitated depression. These studies encouraged the author to examine the possible relationship of the antinicotine effects of a wide variety of CNS agents to their clinical properties. A good relationship was found between blockage of nicotine extensor convulsions and sedative antianxiety properties (Aceto, 1975, accepted for publication in Pharmacology). This relationship was especially good for drugs designated as antidepressants, antipsychotics and antianxiety agents. Because it was shown that for the drugs classified as antianxiety agents, there was a direct relationship between the recommended therapeutic dose in man and antinicotine potency in the mouse, this study will focus on this relationship.

9. Details of experimental design and procedures:

The brain areas which we propose to investigate are the cortex, and cerebellum. The subcellular fractionation procedures described below are currently being used in this laboratory, and are primarily based on those reported by DeRobertis et al., 1962; Mule et al., 1961; and Hokin and Hokin, 1958.

I. SUBCELLULAR FRACTIONATION OF BRAIN TISSUE (DeRobertis et al., 1962; Mule et al., 1967)

Brain tissue is homogenized with a teflon pestle for two minutes at a speed of 400 rpm. The homogenate is diluted with 0.32 M sucrose (Ca++) to give a final concentration of 1 g of brain per 10 ml. An aliquot representing 10% of the total homogenate is removed and labelled homogenate. The remaining homogenate is centrifuged at 900 times g for 10 minutes at 0°C in a Sorvall RC2-B centrifuge. This centrifugation yields a crude nuclear pellet. The supernatant is decanted and the crude nuclear pellet is washed twice. The resulting suspension is then centrifuged at 900 times g for 10 minutes at 0°C. The supernatant is decanted and combined with the other supernatant. The final crude nuclear pellet consists of nuclei, myelin, membrane fractions and tissue debris.

The combined crude nuclear supernatants are centrifuged in the Sorvall RC2-B centrifuge at 11,500 times g for 20 minutes at 0°C to yield a crude mitochondrial pellet which contains mitochondrial nerve endings, membrane fragments and myelin. The supernatant is decanted, and the pellet is washed once with 0.32 M sucrose (Ca++) and centrifuged at 11,500 times g. The pellet wash is added to the supernatant. The final crude mitochondrial pellet is resuspended in a vol-

ume of 0.32 M sucrose equivalent to 1/3 of the original homogenate volume. The combined supernatants are centrifuged for 30 minutes in the Beckman L3-50 ultracentrifuge at 124,000 times g. The supernatant is decanted and referred to as the final soluble supernatant fraction. The pellet, which is the microsomal pellet, is resuspended in 0.32 M sucrose (Ca++) for marker assays or in 0.1 N HCl for radioactive counting.

II. SUBFRACTIONATION OF THE CRUDE MITOCHONDRIA

In order to isolate nerve endings and mitochondria, a discontinuous sucrose density gradient is prepared by layering 0.8 M, 1.0 M, 1.2 M and 1.4 M sucrose in 17-ml cellulose nitrate tubes. An aliquot of the crude mitochondrial fraction is layered on top. The tubes are placed in the Beckman L3-50 ultracentrifuge and spun at 81,000 times g for 120 minutes. The following layers are obtained: myelin, membrane fractions, cholinergic nerve endings, non-cholinergic nerve endings, and free mitochondria.

III. OSMOTIC SHOCK OF NERVE ENDINGS AND ISOLATION OF SYNAPTIC VESICLES

Synaptic vesicles are isolated from nerve endings by diluting an aliquot of the crude mitochondrial (CM) fraction with 10 uM $CaCl_2$ to make a final 0.32 M sucrose solution. The diluted CM is homogenized for 2 minutes at 400 rpm. The homogenate is centrifuged in the Sorvall RC2-B centrifuge at 11,500 times g for 20 minutes at 0°C. The pellet (M_1) consists of swollen mitochondria, myelin fragments, and the subsynaptic web. The supernatant is centrifuged at 124,000 times g for 30 minutes at 0°C. The pellet consists primarily of synaptic vesicles (M_2). The supernatant is considered to be the final soluble supernatant (M_3).

IV. SUBFRACTIONATION OF THE CRUDE NUCLEAR PELLET

In order to isolate pure nuclei, a discontinuous sucrose density gradient with 0.8 M and 1.2 M sucrose is prepared in a 17 ml cellulose nitrate tube by adding 6.0 ml of 1.2 M sucrose and carefully layering 6.0 ml of 0.8 M sucrose on top. 5.0 ml or less of the crude nuclear pellet which has been resuspended in 0.32 M sucrose is then layered on top and spun in the Beckman ultracentrifuge at 81,000 times g at 0°C for 120 minutes. The top layer, designated N_1, consists of large myelin fragments. The middle layer, N_2, consists primarily of nuclei, but contains some mitochondria, myelin fragments and synaptic vesicles. The pellet N_3 contains whole cells, tissue debris and blood cells.

V. ISOLATION OF DNA, RNA AND PHOSPHOLIPID FROM THE SUBCELLULAR FRACTION OF RAT BRAIN

Brain tissue for marker assays is homogenized and subfractionated as described above. Twenty ml of the supernatant and 5-10 ml of each of the remaining subcellular fractions are mixed with an equal volume of 10% TCA in a 45 ml polypropylene centrifuge tube. The samples are centrifuged in the Sorvall RC2-B at 12,000 times g for 5 minutes at 0°C. The liquid is discarded by decantation and the pellets washed four times with cold 5% TCA. Each washing consists of adding cold 5% TCA and resuspending the pellet by gentle stirring with a plastic rod. The suspension is centrifuged at 12,000 times g for 5 minutes and the liquid discarded. To the final pellets are added cold 5% TCA, and the pellets are resuspended by gentle stirring with a plastic stirring rod. One-half of this suspension is removed and placed in a 15 ml polyethylene centrifuge tube for DNA assay. The remaining suspension is centrifuged at 12,000 times g for 5 minutes. The supernatant is decanted and discarded. The pellet

is then suspended in ethanol and $CHCl_3$ is added. The samples are stirred by Vortex until all material dissolves. The tubes are tightly stoppered and stored overnight at 4°C. One then adds 0.1 N HCl (cold) and shakes to form an emulsion. The emulsion is centrifuged in the Sorvall RC2-B at 30,000 times g for 5 minutes at 0°C. The aqueous layer contains the RNA, and the chloroform layer contains the phospholipids.

VI. CHEMICAL AND ENZYMATIC MARKER ASSAYS

In order to verify the morphology of each fraction, the following chemical and enzymatic assays are performed:

1. Protein is determined by the method of Lowry (1951).
2. DNA is estimated by the diphenylamine reaction described by Burton (1956).
3. RNA is estimated by Schneider's RNA assay (1957).
4. Phospholipid phosphorus is determined by the method described by Bartlett (1959).
5. Succinic dehydrogenase activity is determined by the method of Bonner (1955).
6. NADPH-cytochrome-C-reductase activity is determined as described by Mule (1967).

DNA, RNA and phospholipid are isolated for assay as described previously.

VII. DETECTION OF RADIOACTIVITY AND IDENTIFICATION OF NICOTINE

An aliquot of the crude mitochondrial fraction is subjected to osmotic shock, rehomogenization and centrifugation in order to isolate synaptic vesicles. An aliquot of each fraction that contains radioactivity is oxidized in a Packard Tri-Carb Sample Oxidizer. The samples are counted in a Beckman scintillation counter and corrected for quenching by external standardization. To determine how much of the radioactivity is due to unchanged drug or metabolites, thin-layer and gas chromatography of organic extracts is done.

VIII. ANIMAL STUDIES

The research plan is to first determine the disposition of nicotine-^{14}C in male rats. Nicotine-^{14}C will be given intravenously to six naive animals per dose and at least three doses of nicotine will be studied. At 5 and 20 minutes after nicotine-^{14}C, the animals will be sacrificed and the brain levels and subcellular disposition of nicotine will be determined. In the drug studies, animals will also be injected subcutaneously with selected antianxiety agents such as diazepam, chlordiazipoxide and meprobamate (each drug will be given at three dose levels and at two different time periods; namely, ½ and 2 hours) before receiving radioactive nicotine. Saline controls will be run with each drug group and the mean concentration of nicotine-^{14}C in the various brain areas and subcellular fractions will be determined and expressed as pmol/g $\pm$ S.D. or as % of control and an analysis of variance of the data will be done with each drug. These results will be used to interpret the possible involvement and function of the nicotine nervous system as it relates to antianxiety agents. For the localization studies, 50 to 100 mg portions of selected brain areas will be oxidized in the Packard Oxidizer and the radioactivity counted in the Beckmann scintillation instrument. In the subcellular experiments, the selected brain areas will be pooled to yield sufficient tissue for the studies (2 g.).

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Aceto.(1974). Effects of CNS agents on nicotine extensor convulsion and lethality in mice and their sedative-antianxiety effects in man. Accepted for publication by Pharmacology, 1975.

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DeRobertis, C., Pellegrino De Iraldi, A., Rodriquez de Lores A., and Solganicoff, L.. Cholinergic and non cholinergic nerve endings in rat brain-Isolation and subcellular distribution of acetyl chain and acetyl cholinesterase. (1962). J. of Neurochem., 9, 23-35.

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Larson, P.S., and Silvette, Tobacco Experimental and Clinical Studies. A Comprehensive Account of the World Literature. William and Wilkin, Baltimore, 1961, and Supp. I, 1968 and Supp. II, 1971.

Lowry, O.H., Rosenbrough, N.F., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

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Schneider, W.C., (1957). Determination of nucleic acids on Tissues by Pentose Analysis. METHODS OF ENZYMOLOGY, ed. by S.P. Cotowich and N.O. Kaplan, Academic Press, N.Y., N.Y., Vol. 3, 680.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Department of Pharmacology is currently occupying 20,000 square feet of space. The department has a library which makes the important journals available for the members. In addition, a new Health Sciences Library has been built in close proximity to the Department of Pharmacology.

The Department of Pharmacology has its own animal facilities and they have recently been refurnished. Attendents for the care of the animals are supported by the department.

The CNS Division of the Department of Pharmacology occupies a space of 8,000 square feet with office and research space available. These laboratories are well supplied with pharmacological equipment. The proposed study will be conducted in one of these laboratories.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Biographical Sketch

Name: Aceto, Mario D. Role in Project: Principal Investigator

Date of Birth: REDACTED Marital Status: REDACTED

Education: University of Rhode Island, Kingston, R.I. - B.S. Pharmacy -
University of Maryland, College Park, MD. - M.S. Pharmacology - 1955
University of Connecticut, Storrs, Conn. - Ph.D. Pharmacology - 1959

Professional Experience:

1973 -	Associate Professor of Pharmacology, Medical College of VA.
1973 - 1973	Project Head, Sterling-Winthrop Research Institute
1967 - 1973	CNS Section Head and Project Leader, Sterling-Winthrop Research Institute
1966 - 1973	Senior Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1964 - 1966	Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1963 - 1966	Group Leader (Pharmacology) Sterling-Winthrop Research Institute
1964 - 1972	Lecturer in Pharmacology, Albany Medical College
1962 - 1963	Associate Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1959 - 1962	Assistant Professor, University of Pittsburgh
1958 - 1959	Instructor, Pharmacology, University of Pittsburgh
1956 - 1958	Graduate Assistant, University of Connecticut
1953 - 1956	Graduate Assistant, University of Maryland

Honors: Honor Achievement Award granted by the American College of Angiology for the top animal study published during a 5 year period in Angiology in June, 1966. REDACTED

Publications:

Aceto, M.D., Harris, L.S., Dewey, W.L. and Balster, R.L.: Dependence studies of new compounds in rhesus monkeys. Committee on Problems of Drug Dependence, 1975. (In Press).

Aceto, M.D. and Harris, L.S.: Comparative study of the effects of two narcotic antagonists naloxone and nalorphine on developing dependence in rhesus monkeys. Accepted for publication in J. Pharmac. Exptl. Ther. (1975).

Aceto, M.D.: Effects of CNS agents on nicotine extensor conculsions and lethality in mice and their sedative-antianxiety effects in man. Accepted for publication in Pharmacology (1975).

Aceto, M.D., Harris, L.S., Balster, R.L., and Dewey, W.L.: Evaluation of dependence liability of narcotic agonist and antagonist. Committee on Problems of Drug Dependence 77-78, 1974.

Publications (continued)

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Harris, L.S., Pearl, J., and Aceto, M.D.G.: Similarities in effects of barbiturates and mild tranquilizers on activity in mice. Psychon. Sci., 4: 267-268, 1966.

Biographical Sketch

Name: Dewey, William L. Role in Project: Co-Investigator

Date of Birth: REDACTED Martial Status: REDACTED

Education:

St. Bernardine of Siena College, Loudonville, N.Y.	- B.S.	Biology - 1957
College of Saint Rose, Albany, N.Y.	- M.S.	Biology - 1964
Univ. of Connecticut, Storrs, Conn.	-Ph.D.	Pharmacology - 1967

Professional Experience:

1973 -	Associate Professor Dept. of Pharmacology, Medical College of Va., Richmond, Va.
1969 - 1973	Assistant Professor of Pharmacology, University of North Carolina, Chapel Hill, N.C. 27514
1971 - 1973	Consultant, Sharps Associates, 767B Concord Ave., Cambridge, Mass.
1968 - 1969	Instructor of Pharmacology, University of North Carolina, Chapel Hill, N.C. 27514
1967 - 1970	Consultant, Arthur D. Little Inc., Cambridge, Mass.
1967 - 1968	Postdoctoral Research Trainee. Neurobiology Program (MH-1107-01) University of North Carolina, Chapel Hill, N.C.
1966 - 1967	Postdoctoral Research Fellow, Dept. of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, N.C. 27514
1964 - 1966	Graduate Assistant, School of Pharmacy, University of Connecticut
1959 - 1964	Assistant Research Biologist, Sterling-Winthrop Research Institute Rensselaer, N.Y.

Honors: REDACTED

Publications:

Aceto, M.D., Harris, L.S., Dewey, W.L., and Balster, R.L.: Dependence studies of new compounds in the rhesus monkey (Submitted for publication.)

Dewey, William L., Patrick, Graham A. and Harris, Louis S.: Annual Report: Narcotic Antagonists in the rat infusion technique (submitted for publication.)

Dewey, William L. (Book Review) Narcotics and the hypothalamus, Kroc Foundation Symprsia No 2. Zimmerman and Gerge editors. Amer. J. Pharm. Ed. (in press).

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Publications (continued)

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Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.S.: Subcellular and tissue distribution of H3-delta-9-tetrahydrocannabinol in brain and peripheral organs of nontolerant and tolerant dogs (Submitted for publication).

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Martin, B.R., Dewey, W.L., Harris, L.S. and Beckner, J.S.: H3-Delta-9-tetrahydrocannabinol distribution in pregnant dogs and their fetuses (Submitted for publication).

Biographical Sketch

Name: Harris, Louis S. Role in Project: Co-Investigator

Date of Birth: REDACTED Marital Status: REDACTED

Education:

Harvard College, Cambridge, Mass.	- B.S.	Chemistry - 1954
Harvard University, Cambridge, Mass.	- M.S.	Medicinal Sci. - 1956
Harvard University, Cambridge, Mass.	- Ph.D.	Pharmacology - 1958

Professional Experience:

1972 -	Professor and Chairman, Pharmacology, Medical College of Va.
1970 -	Professor of Pharmacology, University of North Carolina School of Medicine, and School of Pharmacy
1969 -	National Institute of Mental Health Psychotomimetic Agents Committee-Chairman, 1971 -
1969 -	Editorial Board, Journal of Pharmacology and Experimental Therapeutics
1966 - 1970	Associate Professor of Pharmacology, University of North Carolina School of Medicine and School of Pharmacy
1962 - 1966	Section Head in Pharmacology and Senior Research Biologist, Sterling-Winthrop Research Institute
1961 - 1962	Associate Member, Sterling-Winthrop Research Institute
1960 - 1961	Research Biologist, Sterling-Winthrop Research Institute
1959 - 1966	Lecturer in Pharmacology, Albany Medical College
1958 - 1960	Research Associate, Sterling-Winthrop Research Institute
1955 - 1958	National Institutes of Health, Fellow
1954 - 1955	National Science Foundation, Fellow
1951 - 1952	Research Assistant in Anesthesiology, Massachusetts General Hospital

Honors: Winner of Martius Yellow Competition - Department of Chemistry, Harvard College, 1958, REDACTED

Publications:

Dewey, W.L., Patrick, G.A., and Harris, L.S.: Annual Report: Narcotic' antagonist in the rat infusion technique. Committee on Problems of Drug Dependence, 1975.

Aceto, M.D., Harris, L.S., Dewey, W.L., and Balster, R.L.: Dependence studies of new compounds in the rhesus monkey. Committee on Problems of Drug Dependence, 1975.

Chiplin, R.E., Dewey, W.L., Harris, L.S., and Lowenthal, W.: Effect of propranolol on antinociceptive and withdrawal characteristics of morphine. Submitted to Pharmacology, Biochemistry and Behavior.

Publications (continued)

Dewey, W.L., Martin, B.R., and Harris, L.S.: Chronic effects of Δ^9-THC in animals: Tolerance and biochemical changes. Submitted

Patrick, G.A., Dewey, W.L., Spaulding, T.C., and Harris, L.S.: Relationship of brain morphine levels to analgesic activity in acutely treated mice and rats and in pellet-implanted mice. Submitted to JPET.

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Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.: Marihuana-like activity of new synthetic tetrahydrocannabinols. In Press to Pharmacology, Biochemistry, and Behavior.

Munson, A.E., Levy, J.A., Harris, L.S., and Dewey, W.L.: Effects of Δ^9-tetrahydrocannabinol on the immune system. International Conference on the Pharmacology of Cannabis, Savannah, Georgia, December 3-6, 1974.

Munson, A.E., Harris, L.S., Friedman, M.A., Dewey, W.L., and Carchman, R.A.: Anti-neoplastic activity of cannabinoids. Submitted to J. National Cancer Institute.

Pedigo, N.W., Dewey, W.L., and Harris, L.S.: Determination and characterization of the antionociceptive activity of intraventricularly administered acetylcholine in mice. In Press to JPET.

Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.S.: Subcellular localization H^3-Δ^9-tetrahydrocannabinol in brain of nontolerance and tolerant dogs (Submitted to JPET).

14. First year budget.

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
Aceto, Mario D.	Principal Investigator	25	
Dewey, William L.	Co-Investigator	5	
Harris, Louis S.	Co-Investigator	.1	
Technical			
To be recruited, Lab Specialist + 10.06 fringe benefits		100	REDACTED
		Sub Total for A	

B. Consumable supplies (by major categories)

Animals and animal care; chemicals; radiochemicals; glassware, etc.

Sub-Total for B 8,000.00

C. Other expenses (itemize)

Travel (Fed. Am. Soc. Expt. Biol.) - 350.00

Misc., Laundry, reprint charges, office supplies, maintenance of equipment -- 1000.00

Sub-Total for C 1,350.00

Running Total of A + B + C — REDACTED

D. Permanent equipment (itemize)

Beckman Liquid Scintillation LS 330 System (Dept. has an instrument, but demands on its use are heavy)

Sub Total for D 14,500.00

E. Indirect costs (15% of A+B+C) 3,301.00

Total request 39,808.00

15. Estimated future requirements:

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	REDACTED	8,000	1,350	--	3,509	REDACTED
Year 3	--	--	--	--	--	--

*Includes 1,399 fringe benefits.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Nicotine Nervous System Interaction with CNS Drugs	A.D. Williams Research Award (seed money)	1,600	11/75 - 11/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Mechanism Studies on Experimental Brain Edema		232,107	9/1/75 - 8/31/80

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Mario D Aceto

Signature Mario D Aceto Date 6/27/75

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Title Associate Vice President
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